Journal of Medical Virology 61:228–234 (2000)

Expression of Caspid Protein VP1 for Use as Antigen for the Diagnosis of Enterovirus 71 Infection

Shin-Ru Shih,[1] Yi-Shuane Li,[1] Chiuan-Chian Chiou,[1] Pin-Chau Suen,[5] Tzou-Yien Lin,[2] Luan-Yin Chang,[2] Yhu-Chering Huang,[2] Kuo-Chien Tsao,[3] Hsiao-Chen Ning,[3] Tzong-Zeng Wu,[4] and Err-Cheng Chan[1]*

[1]*School of Medical Technology, Chang Gung University, Taiwan*
[2]*Department of Infectious Diseases, Chang Gung Children's Hospital, Taiwan*
[3]*Clinical Virology Laboratory, Institute of Biotechnology, Taiwan*
[4]*Institute of Biotechnology, National Dong Hwa University, Taiwan*
[5]*Department of Clinical Pathology, and Department of Dentistry, Chang Gung Memorial Hospital, Taoyuan, Taiwan*

To produce enterovirus 71 antigen for diagnostic purposes, the gene encoding the entire capsid protein VP1 was amplified by reverse transcription-polymerase chain reaction (RT-PCR), cloned and expressed in *Escherichia coli* as a polyhistidine fusion protein. Western blotting experiments with sera from patients with enterovirus 71 infection indicated that immunoglobulin G (IgG) and IgM antibodies bound to a single polypeptide VP1. According to these results, IgM anti-VP1 appeared in sera of patients with a symptomatic enterovirus 71 acute infection, whereas IgG anti-VP1 was present in sera of past infection. This finding suggests that detecting IgG and IgM immune responses against linear epitopes of recombinant VP1 is an effective means of determining the different phases of enterovirus 71 infection. In addition, sera containing coxsackie virus 16 (CA16) antibodies did not cross-react with the recombinant VP1 of enterovirus 71, despite the homology between VP1 proteins of both viruses. Comparison with reference PCR and neutralization assays showed these antibody tests to be appropriate for the serodiagnosis of enterovirus 71 infection. ***J. Med. Virol. 61:228–234, 2000.***

KEY WORDS: enterovirus 71; recombinant capsid protein VP1; serodiagnosis; acute infections; past infections; enterovirus 71 antibody

INTRODUCTION

Enterovirus 71 is a member of the family *Picornaviridae*, and its genome is a single stranded positive sense RNA. Enterovirus 71 has a capsid consisting of four proteins, VP1 to VP4. Variation of capsid proteins, except VP4, are responsible for the antigenic diversity among the enteroviruses; neutralization epitopes reside mainly on VP1 [Rueckert, 1990]. Enterovirus 71 and coxsackie virus A16 are two major etiological agents of hand, foot and mouth disease in children [Hagiwara et al., 1978]. Generally, infection with enterovirus 71 is considered to be asymptomatic or only causes a mild hand, foot and mouth disease. In some cases, however, enterovirus 71 infection causes disorders of the central nervous system (CNS) such as aseptic meningitis/encephalitis [Blomberg et al., 1974] and acute flaccid paralysis [Schmidt et al., 1974]. Several investigations have suggested that the different clinical patterns associated with enterovirus 71 are possibly due to a difference in pathogenicity of the associated viral strains [Chang LY et al., 1998; Hagiwara et al., 1984; Ishimaru et al., 1980; Melnick JL, 1984]. Outbreaks of enterovirus 71 associated with diseases of CNS have been reported worldwide [Blomberg et al., 1974; Chonmaitree et al., 1981; Gilbert et al., 1988; Kennett, M et al., 1974; Nagy et al., 1982; Samuda et al., 1987]. Since April 1998, hand, foot and mouth disease or diseases of CNS have affected many children in Taiwan, with over eighty fatalities. Some autopsy results from the cases of this outbreak indicated the presence of enterovirus 71 nucleic acids and enterovirus 71 antigens in brain tissue or body fluids. No effective antiviral drug for enterovirus 71 is available. Early diagnosis, however, would limit the spread of this virus

Grant sponsor: National Science Council, ROC; Grant number: NSC-88-2314-B-182-064; Grant sponsor: National Health Research Institute, ROC; Grant number: NHRI-CN-CR8803P.

*Correspondence to: E.-C. Chan, School of Medical Technology, Chang Gung University, 259 Wen-Hua 1st Road, Kwei-Shan, Taoyuan 333, Taiwan. E-mail: chanec@mail.cgu.edu.tw

Accepted 12 November 1999

and reduce the mortality. Furthermore, in addition to coxsackie 16 and 10, enterovirus 71 is a known causative agent of hand, foot and mouth disease and it is the only one associated with significant mortality due to the involvement of the CNS. Early diagnosis of enterovirus 71 would allow distinction between enterovirus 71 and coxsackie virus infection, and permit special care to reduce complications and mortality.

Several methods are available for the diagnosis of enterovirus 71 infection, such as the neutralization assay [World Health Organization, 1988] and direct isolation of the virus from body fluids of infected patients and subsequent identification by PCR with strain-specific primers [Zheng et al., 1995] or by immunofluorescent staining with a monoclonal antibody. The above methods, however, require virus culture, which is time consuming and tedious. Therefore, this study focused on obtaining a recombinant VP1 antigen to establish a rapid serological test to screen for enterovirus 71, and the diagnostic value of this recombinant protein was assessed.

MATERIALS AND METHODS

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) of VP1

Monkey kidney cell-line (LLC-MK2) was used for enterovirus culture as described elsewhere [Zheng et al., 1995]. Total DNA-free RNA was extracted from the prototype enterovirus 71 BrCr-infected LLC-MK2 cells using TRIzol reagent-chloroform (Gibco Inc., USA) extraction as described elsewhere [Chomczynski and Sacchi, 1987]. The concentration of RNA was determined spectrophotometrically at 260/280 nm and quality as assessed by visualization on an ethidium stained agarose gel. RT-PCR for cDNA synthesis and amplification was performed in a single step by adding RNA template and primers to RT-PCR beads (Amersham Pharmacia Biotech, Buckinghamshire, UK) containing M-MuLV reverse transcriptase, RNase inhibitor, buffer, nucleotides, and *Taq* DNA polymerase.

Expression and Purification of VP1 Protein

Two PCR oligodeoxynucleotide primers were synthesized according to the GenBank Nucleotide Sequence Database under the accession number U00871 (E71 BrCr). A NdeI and a *Bam*HI restriction endonuclease site in primers (underlined below) were then introduced to allow ligation of the entire VP1 cDNA into pET14b, a prokaryotic expression vector containing an amino-terminal histidine tag (Novagen Inc., Madison, WI). The primer sequences were 5′-AAGGAAG-CATATGGACAGAGTGGCAGAT GTGAT-3′ and 5′-GTATAGGATCCCCCGAGCGTAGTGATTG-3′. PCR amplification was then carried out in a DNA Thermal Cycler (Perkin-Elmer, GeneAmp 9600) in sequential cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min with an extension step for 25 cycles. The correct PCR product was 891-bp in length and was verified by DNA sequencing and restriction endonuclease digestion before expression. After introducing the constructed plasmid into *E. coli* BL21 (DE3 pLysS), the expression of His-VP1 fusion protein was induced with 40 μM isopropyl β-D-thiogalactopyranoside (IPTG) as described elsewhere [Studier et al., 1990; Rosenberg et al., 1987]. Inclusion body containing the fusion protein was dissolved in 6 M urea and refolded by slowly diluting into a pH 7 buffer as described elsewhere [Lowe et al., 1987]. To purify the expressed fusion protein, the refolded VP1 protein solution was filtered through a 0.45 μm-pore-size filter and applied to a nickel affinity chromatography column (Novagen) according to the manufacturer's instructions. Purity of the recombinant VP1 was analyzed by SDS-PAGE (12% acrylamide), followed by staining with Commassie Brilliant Blue and scanned by a densitometer (Bio-Rad Laboratories, Hercules, CA).

Human Sera Samples

The sera samples from symptomatic cases of enterovirus 71 or coxsackie virus A16-infected patients were received in the Clinical Virology Laboratory, Chang Gung Memorial Hospital, Taiwan. Sera from cases confirmed by virus culture and neutralization test were collected for the immunoblot study. Virus culture was identified by RT-PCR with enterovirus 71 specific primers as described elsewhere [Zheng et al., 1995]. The neutralization assay was performed in human rhabdomyosarcoma (RD) cell according to the standard procedure described elsewhere [World Health Organization, 1988]. The serum samples included 26 sera from cases of past enterovirus 71 infection, 12 sera from patients with acute enterovirus 71 infection, 2 sera from patients with acute coxsackie virus A16 infection, and 6 sera from patients with acute coxsackie virus A16 with past enterovirus 71 infection. Six sera from persons with past infection with enterovirus 71 were negative by the neutralization test. Table I lists the general patient characteristics of acute infection cases. Control sera were collected from 5 healthy blood donors negative for neutralization test and without IgG and IgM immune responses against enterovirus 71 and coxsackie virus A16.

Western Blot Analysis

Proteins were dissolved in SDS-PAGE sample buffer and then heated at 95°C for 5 min. The denatured proteins were subjected to SDS-PAGE (12% polyacrylamide) and transferred to a PVDF membrane (Millipore, Bedford, MA) by electroblotting for 1 hr at 1 Amp. The blotted PVDF membrane was cut into strips and incubated in a blotting solution (Tris-buffered saline, 1% Tween 20, 1% gelatin, 5% skim milk) for at least 1h at room temperature and, then, incubated with the individual serum. Blotted membrane strips were washed three times in TBS buffer, 1% Tween 20, and bound antibody was detected using the alkaline phosphatase-conjugated polyclonal anti-human IgG or IgM (Biogenesis Inc., Sandown, NH) as described elsewhere [Lin et al., 1998]. All serum samples were diluted 1:1,000 be-

TABLE I. Patient Characteristics of Acute EV71 and CA16 Infections*

Case	Age (year)/ gender	Symptom	Virus culture test	Neutralization test for EV71 antibody
1	2/M	HFMD[a]	EV71	+++
2	5/F	HFMD	EV71	+++
3	1/M	HFMD/Encep[b]	EV71	++
4	1/M	HFMD	EV71	+++
5	2/F	HFMD	EV71	+
6	3/M	Herp/Encep	EV71	+
7	4/M	HFMD	EV71	+++
8	2/F	HFMD	EV71	++
9	5/M	HFMD/CNS[c]	EV71	++
10	2/F	HFMD	EV71	+++
11	1/M	HFMD/Meningitis[d]	EV71	++
12	3/M	HFMD	EV71	+
13	3/M	HFMD	CA16(ev71)[e]	—
14	3/M	HFMD	CA16	—
15	2/F	HFMD	CA16(ev71)	—
16	2/F	HFMD	CA16(ev71)	—
17	5/F	HFMD	CA16(ev71)	—
18	2/M	HFMD	CA16	—
19	4/M	HFMD	CA16(ev71)	—
20	4/M	HFMD	CA16(ev71)	—

*The interval between onset of symptoms and serum collection for the acute infection was within 5 days.
[a]HFMD: hand-foot-and mouth disease.
[b]Encep: Encephalomyelitis.
[c]CNS: central nervous system disease.
[d]Meningitis: Aseptic meningitis.
[e]ev71: past EV71 infection.

fore use. Each membrane strip contained 0.25 μg VP1 protein.

RESULTS

Expression of Enterovirus 71-Encoded VP1 Protein

To produce the purified VP1 protein, a full-length VP1 DNA was expressed in *E. coli* using the expression vector pET14b with IPTG induction. The VP1 was produced as a fusion protein with a six-histidine repeat to its amino terminus. In the SDS-PAGE analysis, a band with an approximately 40 kDa, corresponding to the expected molecular mass of VP1 fusion protein was detected in an IPTG-inducible manner (Fig. 1A). The sera from patients with enterovirus 71 infection recognized the 40 kDa in the Western blot analysis (Fig. 1B). The expression level of VP1 protein was estimated as 25% of the total *E. coli* protein by scanning the density of band profiles in the SDS gel. The recombinant VP1 protein was produced as a form of inclusion body in the transformed *E. coli.* The insoluble VP1 protein could be dissolved in a 8 M (pH 7.0) urea solution, and refolding the denatured VP1 protein by diluting into a phosphate buffer (pH 7.0) resulted in a higher VP1 recovery yield than by a dialysis procedure. The 40 kDa VP1 protein was purified to 95% homogeneity by a nickel-resin column (Fig. 1A) for the following immunoblot experiments.

Human IgG and IgM Responses of Patients Infected With Enterovirus 71 to Recombinant VP1

The potential use of VP1 protein as a diagnostic marker for enterovirus 71 infection was examined. The purified VP1 was tested using Western blot analysis with 42 sera from humans in different phases of enterovirus 71 infection and 5 sera from negative control persons (Fig. 2A,B, representative results). The interval between onset of symptoms and serum collection for the acute infection cases was within 5 days. Table II summarizes the Western blot analysis results. All the sera from 26 patients previously infected with enterovirus 71 were positive for IgG, but negative for IgM against the fusion VP1. Among those serum samples, 6 sera were negative for neutralizing enterovirus 71. This finding suggested that VP1-specific IgG test is more sensitive than the neutralization assay for enterovirus 71 infection. All the serum samples from 12 patients with a symptomatic acute infection with enterovirus 71 had IgM against the VP1 fusion protein. In general, the presence of rheumatoid or antinuclear factor in the sera tested would cause a false positive IgM response. All the sera tested in this study, however, were negative for the rheumatoid factor and antinuclear factor. From above data, it is concluded that detection of IgG and IgM immune responses against the VP1 fusion protein can facilitate the determination of different phases of enterovirus 71 infection.

Cross-Reaction of Anti-Enterovirus 71 and Coxsackie Virus 16 Antibodies

Our previous study indicated that the VP1 nucleotide sequence of enterovirus 71 differs distinctly from that of coxsackie virus 16. Both virus strains, however, exhibited 67% amino acid identity in VP1 sequence using the multiple sequence analysis program of the GCG package. The cross-reactivity of antibodies against the

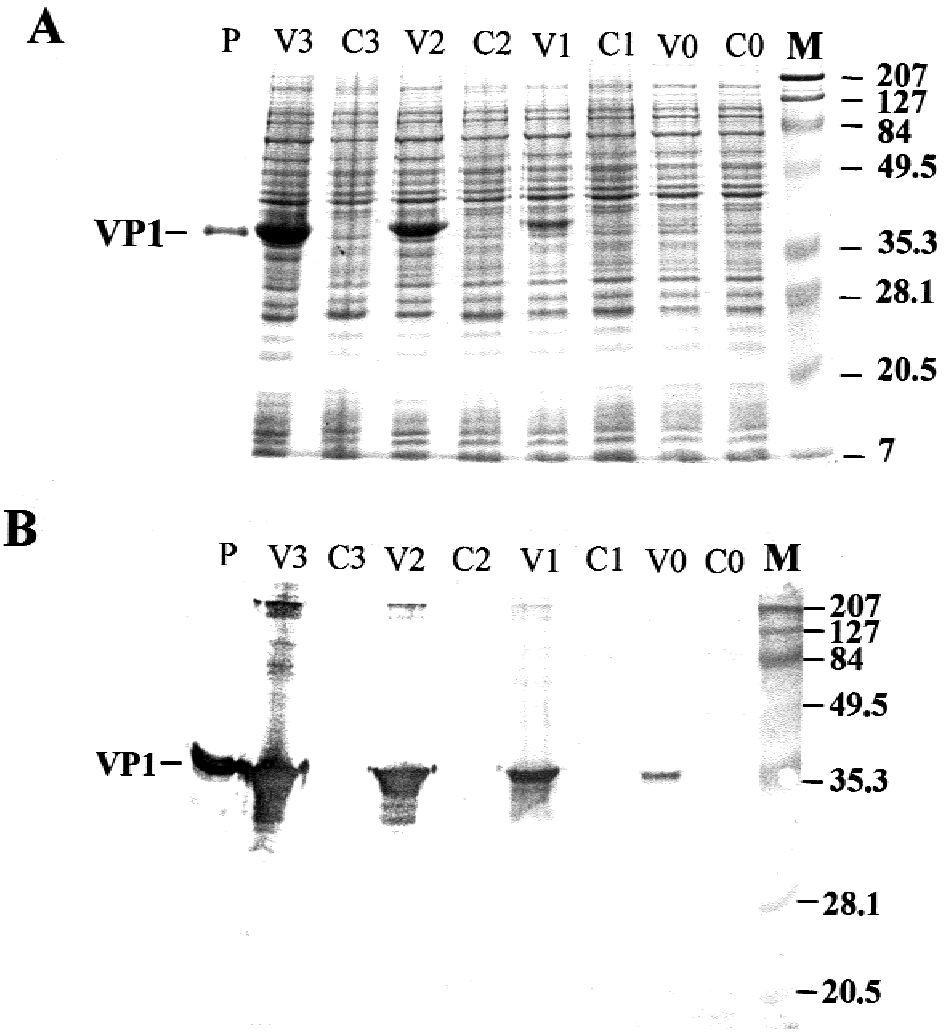

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% acrylamide) and immunoblot of recombinant VP1. (**A**) **Lanes C** denote the proteins from cells of *E. coli* BL21 harboring pET14b as controls, and **lanes V** represent the proteins from cells of *E. coli* BL21 harboring VP1 fused-plasmid. The numbers (0–3) are cells harvested at 0, 1, 2, and 3 hr after IPTG induction. **Lane P** and **lane M** denote the purified recombinant VP1 from nickel affinity chromatography and molecular weight standards, respectively. (**B**) An identical SDS gel as As without Coomassie blue staining was immunoblotted with enterovirus 71 IgG-positive human serum. The denotation is the same as in As.

VP1 of coxsackie virus 16 and enterovirus 71 was examined. The purified recombinant VP1 was tested by Western blot analysis using human sera containing a high titre of anti-coxsackie virus A16 IgG or IgM. Experimental results indicated that, although the sera from the patients with acute coxsackie virus 16 had a high titre of IgM and IgG, these sera did not react with the recombinant fusion protein VP1 (Fig. 3A, B). In the sera of patients with acute coxsackie virus 16 infection, the IgG antibodies reacting with the VP1 fusion protein was due to the past infection with enterovirus 71 (Fig. 3B).

DISCUSSION

The aim of this study was to clone and express the VP1 protein of enterovirus 71 in *E. coli*, and to develop an efficient, rapid and inexpensive diagnostic kit for this infection. The VP1 fusion protein with a histidine tag had an estimated molecular mass of 40 kDa, and was produced by *E. coli* with IPTG induction. Western blot analysis revealed that the recombinant VP1 reacted with sera from patients with enterovirus 71 infection, but not that from uninfected persons, thus confirming the presence of antibody to VP1 in infected sera. Using purified recombinant VP1 as an antigen, the response of antibodies IgG or IgM correlated well with the clinical grouping of the patients and the neutralization assay result. It was also demonstrated that detection of anti-VP1 IgG or IgM has a better sensitivity than neutralization assay for diagnosing enterovirus 71 infection. Using immunoassay method to detect specific IgM antibody commonly leads to false positive results when rheumatoid factor or antinuclear factor are present in sera. Therefore, IgM detection of early infectious diseases is a problem because transient production of rheumatoid factor is often observed at the

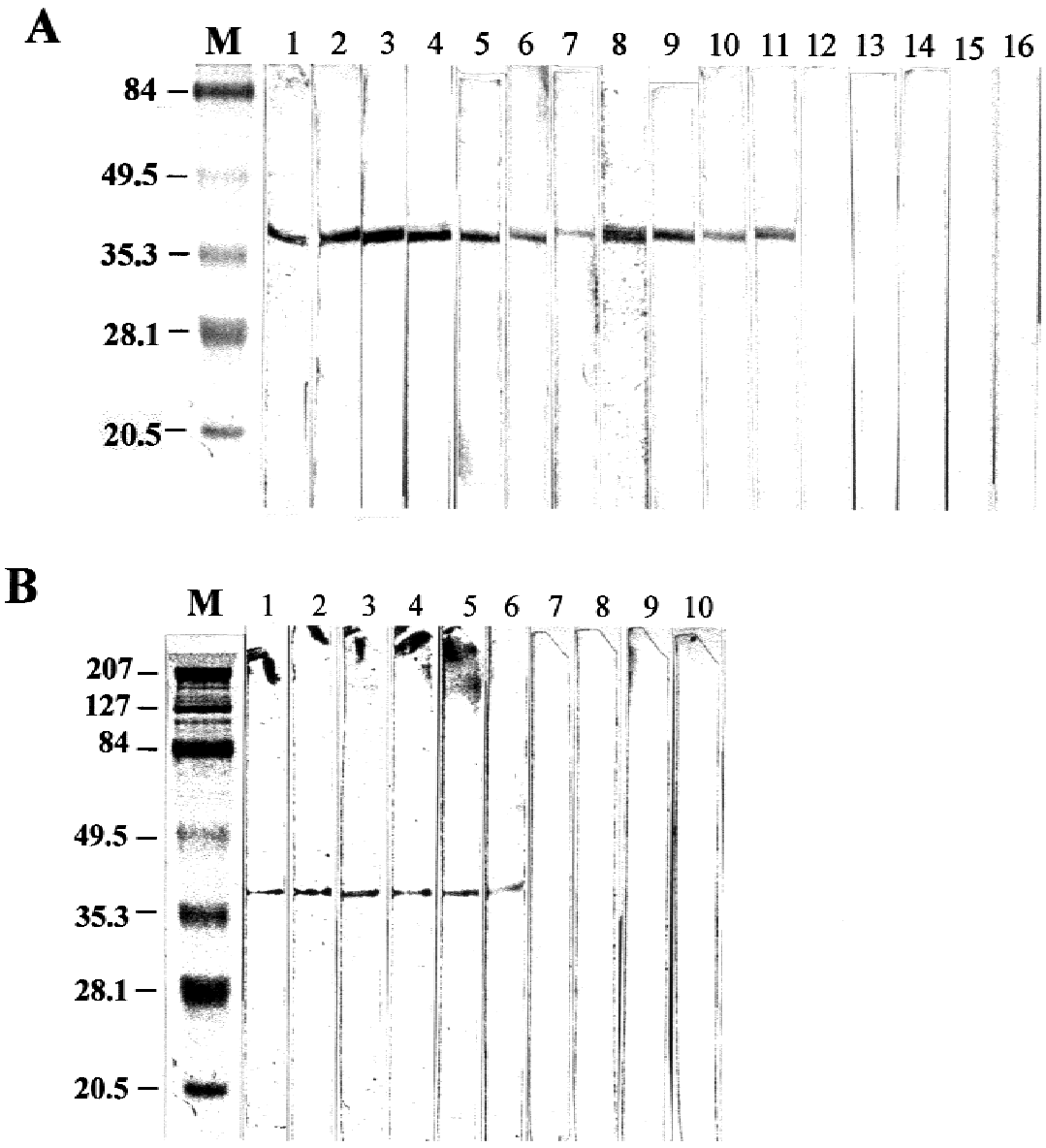

Fig. 2. Western blot analysis for recombinant VP1 antigen using sera from patients with enterovirus 71 infection and healthy persons. Each lane loaded with 0.2 μg purified VP1. (**A**) Western blot assay using goat anti-human IgG antibodies as secondary antibody. **Lanes 1–11** represent immunoblotting with sera from patients with past enterovirus 71 infection, and **lanes 12–16** represent immunoblotting with sera from normal individuals. (**B**) Western blot assay using goat anti-human IgM antibodies as secondary antibody. **Lanes 1–6** represent immunoblotting with sera from patients with a symptomatic enterovirus 71 acute infection, and **lanes 7–10** represent immunoblotting with sera from healthy individuals.

beginning of many infections. Although no such factors were detected in the patients of this study on a routine screening of IgM response to enterovirus 71, the pretreatment of sera should be applied to prevent rheumatoid factor or antinuclear factor binding, namely to saturate the sample with aggregated IgG or *staphylococcal* protein A.

Although 67% amino acid sequence of VP1 from enterovirus 71 is identical to that of coxsackie virus 16, experimental results indicated that antibodies present in sera with coxsackie virus 16 infection did not react with the recombinant VP1. This finding implies that either enterovirus 71 antibody is not present in the coxsackie virus 16 positive sera or the antigenic epitopes of VP1 from both enteroviruses differ from each other; however, the sera used in this study were taken from children under six years old, and older children and adults may have cross-reacting IgG and IgM antibodies owing to heterologous enterovirus infections. This assumption needs to be clarified in a future study.

The results of this study suggest that the recombinant VP1 protein of enterovirus 71 seems to be appropriate as an antigen for the serodiagnosis of this infection. Several factors should be considered and evaluated, however, before applying this recombinant VP1 for diagnosis. The following issues are relevant: (1) panels of sequential follow-up sera from acute-infected patients should be used to evaluate whether or not IgM

TABLE II. Enterovirus 71 and Coxsackie Virus 16

Phase of infection	Number of sera examined	Number of positive serum samples			
				Anti-EV71 VP1	
		Culture test[a]	Neutralization test	IgG	IgM
Acute infection (EV71)	12	12	12	12 (100%)	12 (100%)
Past infection (EV71)	26	0	20	26 (100%)	0 (0%)
Non-infection	5	0	0	0 (0%)	0 (0%)
Acute infection (CA16)	2	0	0	0	0
Acute infection (CA16) with past EV71 infection	6	0	0	6	0

[a]Virus culture was identified by RT-PCR and immunofluorescent stain.
EV71: enterovirus 71; CA16 coxsackie virus 16.

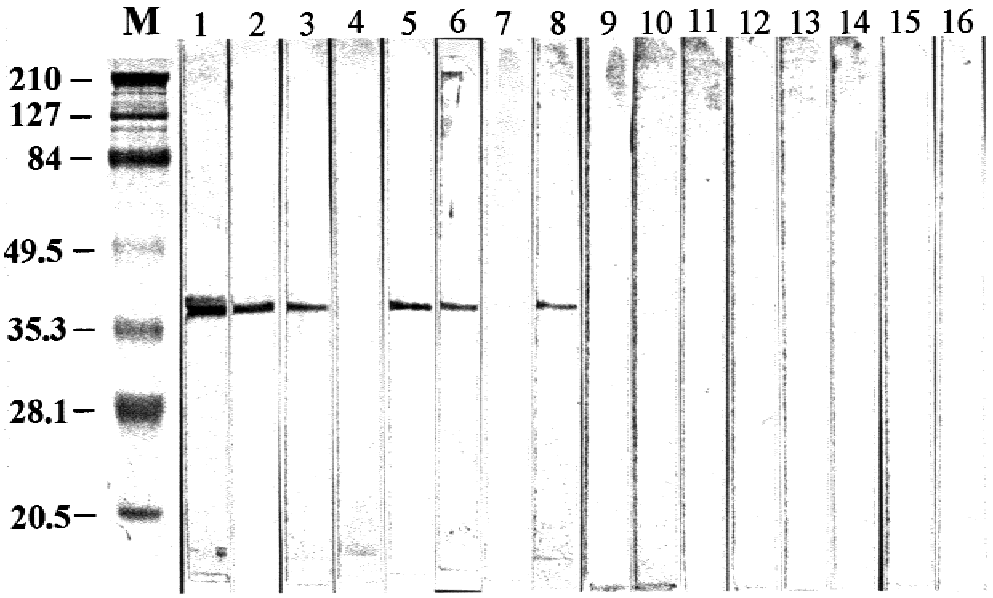

Fig. 3. Western blot analysis for recombinant VP1 antigen using sera containing a high titer of anti-coxsackie virus 16 antibodies. **Lanes 1–3**,**5**,**6**, and **8** were immunoblotted with sera from patients with acute coxsackie virus 16 infection and past enterovirus 71 infection, and **lanes 4** and **7** were immunoblotted with sera from patients with acute coxsackie virus 16 infection using goat anti-human IgG antibodies as secondary antibody. **Lanes 9–16** were immunoblotted with sera from patients with acute coxsackie virus A16 using goat anti-human IgM antibodies as the secondary antibody.

response of the recombinant VP71 persists too long to complicate the interpretation for acute enterovirus 71 infection; (2) a large panel of sera will be used to determine whether or not recombinant VP1 cross-reacts with sera from children infected with other enteroviruses; and (3) sera from a wider population than young children will be used to study the cross-reacting antibodies and persisting IgM responses.

REFERENCES

Blomberg J, Lycke E, Ahlfors K, Johnson T, Wolontis S, von Zeipel G. 1974. New enterovirus type associated with epidemic of aseptic meningitis or hand, foot, and mouth disease. Lancet 2:112.

Chang LY, Huang YC, Lin ZY. 1998. Fulminant neurogenic pulmonary edema with hand, foot, and mouth disease. Lancet 352:367.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Chonmaitree T, Menegus MA, Schervish-Swierkosz EM, Schwalenstocker E. 1981. Enterovirus 71 infection: report of an outbreak with two cases of paralysis and a review of the literature. Pediatrics 67:489–493.

Gilbert GL, Kickson EE, Waters MJ, Kennett ML, Land SA, Sneddon M. 1988. Outbreak of enterovirus 71 infection in Victoria, Australia, with a high incidence of neurologic involvement. Ped Infect Dis 8:611–616.

Hagiwara A, Tagaya I, Yoneyama T. 1978. Epidemic of hand, foot and mouth disease associated with enterovirus type 71 infection. Inter Virol 9:60–63.

Hagiwara A, Yoneyama T, Takami S, Hashimoto I. 1984. Tenotic and phenotypic characteristics of enterovirus 71 isolates from patients with encephalitis and with hand, foot and mouth disease. Arch Virol 79:273–283.

Ishimaru Y, Nakano S, Yamaoka K, Takami S. 1980. Outbreaks of hand, foot, and mouth disease by enterovirus 71. Arch Dis Child 55:583–588.

Kennett M, Birch CJ, Lewis FA, Yung AP, Locarnini SA, Gust ID. 1974. Enterovirus type 71 infection in Melbourne. Bull WHO 51: 609–615.

Lin YL, Liu JS, Chen KT, Chen CT, Chan EC. 1998. Identification of neutral and acidic sphingomyelinase in *Helicobacter pylori*. FEBS Lett 423:249–253.

Lowe PE, Rhind SK, Sugrue R, Marston FAO. 1987. Solubilization, refolding and purification of eukaryotic proteins expressed in *E. coli*. In: Alan R, editor. Protein purification, micro to macro. New York: Liss Inc. p 429–442.

Melnick JL. 1984. Enterovirus type 71 infections: a varied clinical patients sometimes mimicking paralytic poliomyelitis. Rev Infect Dis 6:387–390.

Nagy G, Takatsy S, Kukan E, Mihaly I, Domok I. 1982. Virological diagnosis of enterovirus type 71 infections: experiences gained during an epidemic of acute CNS diseases in Hungary in 1978. Arch Virol 71:217–227.

Rosenberg AH, Lade BN, Chui DS, Lin SW, Dunn JJ, Studier FW. 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. Gene 56:125–135.

Rueckert RR. 1990. Picornaviruses and their replication. In: Fields BN, Knipe DM, editors. Virology. New York: Lippincott-Raven. p 507.

Samuda GM, Chang WK, Yeung CY, Tang PS. 1987. Monoplegia caused by enterovirus 71: an outbreak in Hong Kong. Ped Infect Dis 6:206–208.

Schmidt NJ, Lennette EH, Ho HH. 1974. An apparently new enterovirus isolated from patients with disease of the central nervous system. J Infect Dis 129:304–309.

Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Meth Enzymol 185:60–89.

World Health Organization. 1988. Procedure for using the lyophilized LBM pools for typing enterovirus. Geneva: WHO.

Zheng ZM, He PJ, Caueffield D, Neumann M, Specter S, Baker CC, Bankowski MJ. 1995. Enterovirus 71 isolated from China is serologically similar to the prototype E71 BrCr strain but differs in the 5′-noncoding region. J Med Virol 47:161–167.